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In Vivo Tumor Targeting of a Recombinant Single-Chain Antigen-Binding Protein

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We describe here the first in vivo targeting of tumors with a single-chain antigen-binding protein. The molecule, which was constructed and expressed in *Escherichia coli*, is a novel recombinant protein composed of a variable light-chain (V_L), amino acid sequence of an immunoglobulin tethered to a variable heavy-chain (V_H) sequence by a designed peptide. We show that this protein, derived from the DNA sequence of the variable regions of the antitumor monoclonal antibody B6.2, has the same in vitro antigen-binding properties as the B6.2 Fab' fragment. Comparative pharmacokinetic studies in athymic mice demonstrate much more rapid alpha and beta phases of plasma clearance for the single-chain antigen-binding protein than for the Fab' fragment, as well as an extremely rapid whole-body clearance. Half-life values for alpha and beta phases of single-chain antigen-binding protein clearance were 2.4 minutes and 2.8 hours, respectively, versus 14.8 minutes and 7.5 hours for Fab'. Furthermore, the single-chain antigen-binding protein molecule did not show accumulation in the kidney as did the Fab' molecule or, as previously shown, the $F(ab')_2$ molecule. Despite its rapid clearance, the single-chain antigen-binding protein showed uptake in a human tumor xenograft comparable to that of the Fab' fragment, resulting in tumor to normal tissue ratios comparable to or greater than those obtained with the Fab' fragment. These studies thus demonstrate the in vivo stability of recombinant single-chain antigen-binding proteins and their potential in some diagnostic and therapeutic clinical applications in cancer and other diseases. [J Natl Cancer Inst. 82:1191-1197, 1990]

Clinical applications of monoclonal antibodies (MAbs) in cancer management include both diagnostic and therapeutic modalities. Diagnostic applications include the administration of radiolabeled MAbs for the detection of occult primary or metastatic disease via gamma scanning (1) or the use of an intraoperative gamma-detecting hand-held probe (2). One element in these applications is the availability of MAbs or MAb fragments that will clear from plasma rapidly, resulting in higher tumor to

normal tissue ratios postadministration. Therapeutic applications with MAbs to date have included both native MAb and MAb bound to drugs or radionuclides. One major problem that has emerged with the therapeutic use of radiolabeled MAbs is that the radiolabeled MAb not bound to tumor remains in the circulation with a half-life ($T_{1/2}$) of several days, resulting in potential bone marrow toxicity (3-5). Thus, an MAb form that clears the blood pool more rapidly would be advantageous. Another concern with the use of large molecules such as immunoglobulins as therapeutic agents is their inability to penetrate large tumor masses; a smaller MAb form would be expected to exhibit better penetration. Finally, in all diagnostic and therapeutic applications, a major problem has been the appearance of the human anti-murine antibody (HAMA) response. This response has been shown to arise in approximately 50% of patients after one MAb administration and in more than 90% of patients after two or three MAb administrations (6,7). While $F(ab')_2$ and/or Fab' fragments help to reduce the HAMA response (6,7), it is often difficult to generate these immunoglobulin (Ig) forms in a manner that retains their immunoreactivity in vivo.

Recent advances in technology involving the cloning of Ig genes, the generation of recombinant/chimeric Ig genes, and their expression in a variety of systems promise to be extremely important, not only in delineating structure-function relationships of the Ig molecule, but also in clinical applications. One of these advances has been the development of recombinant single-

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chain antigen-binding proteins (SCAs) (8-10), composed of a variable light-chain amino acid (V_L) sequence of an Ig tethered to a variable heavy-chain (V_H) sequence by a designed peptide that links the carboxyl terminus of the V_L to the amino terminus of the V_H or vice versa. Several SCAs, also termed single-chain Fvs (8), have recently been constructed and expressed in *Escherichia coli* and, using a variety of linkers, have been reported to have K_a values approximately one third to one seventh that of the Fab or Fab' fragments (8,9), and in one case, one third that of the intact IgG (10). These studies also demonstrate the potential importance of the individual linkers, used to tether the V_H and V_L chains, in the binding properties of SCAs. To date, however, no SCA has been analyzed in vivo for stability, pharmacokinetic properties, and the ability to bind a target such as a tumor. Indeed, one concern was that due to the small size of the SCA molecule (25 vs. 50 kilodaltons for Fab', 100 kilodaltons for F(ab')₂, and 150 kilodaltons for intact IgG), it would leave the bloodstream so fast that there would be insufficient time for tumor targeting. Moreover, due to the tethering of the V_H and V_L sequences by a designed amino acid linker, it was unknown whether this type of molecule would maintain its antigen-binding capability in vivo.

Materials and Methods

MAB B6.2 IgG

MAB B6.2 IgG was generated by the immunization of BALB/c mice with a membrane-enriched fraction of a human breast tumor metastasis to the liver (11). MAB B6.2 recognizes a 90-kilodalton glycoprotein found on the surface of many human carcinomas as well as some normal tissues including human granulocytes (12,13).

B6.2 IgG was purified from ascitic fluid by ammonium sulfate precipitation followed by ion-exchange chromatography (DE52; Whatman, Hillsboro, Ore) as previously described (14). The appropriate fractions were pooled and dialyzed against phosphate-buffered saline prior to size-exclusion chromatography (Ultrogel 44; Pharmacia LKB, Bromma, Sweden). The protein concentration was determined by the method of Lowry et al. (15).

Fab' fragments were generated by the digestion of the purified IgG with pepsin as previously described (14). Briefly, B6.2 IgG was incubated with 10 mM 1,4-dithiothreitol and then with iodoacetamide to block the thiol groups. The IgG was then digested with pepsin (1%) at 37 °C for 16 hours. The Fab' fragments were then purified by size-exclusion chromatography. F(ab')₂ fragments were similarly prepared without 1,4-dithiothreitol or iodoacetamide treatment.

Single-Chain Antibody Preparation

The B6.2/212 SCA gene was constructed by combining a synthetic V_L region sequence (16) and a complementary DNA copy of the V_H region sequence¹ via a linker sequence designated linker 212. This linker sequence is similar to, but slightly longer than, a previously published linker sequence (9). In an SCA protein based on the antiluorescein antibody 4-4-20, the 212

linker allowed an increase in affinity.² A *Hind*III site was placed near the 3' end of the V_L and an *Afl* II site near the 5' end of the V_H segment to introduce the linker. An *Aat* II site was placed at the 5' end of the SCA gene and used to generate a fusion to the signal peptide from the *E. coli* ompA gene. Expression of the resulting gene was achieved in *E. coli* with a hybrid lambda promoter (O_L/P_R). Plasmid pGX4663, in which the gene is transcribed from the O_L/P_R promoter, was introduced into *E. coli* strain N99 (c1857). Expression was introduced by temperature shift from 30 °C to 42 °C. The B6.2/212 protein was expressed at 5%-10% of total cell protein. The resulting correctly processed B6.2/212 protein was insoluble but did not appear as inclusion bodies.

Insoluble protein was recovered after expression and cell lysis. This protein was solubilized in 50 mM Tris (pH 8.0), 6 M guanidine-HCl, 50 mM KCl, and 0.1 mM phenylmethyl sulfonyl fluoride. Renaturation was achieved by dilution (1:200) into the same buffer without guanidine. The renatured SCA protein was purified by cation exchange chromatography.

Labeling of B6.2 IgG and Fragments

MAB B6.2 IgG, Fab', and SCA were labeled with Na¹²⁵I or Na¹³¹I using Iodo-Gen (Pierce Chemical, Rockford, Ill) (14). Twenty micrograms of IgG or 100 µg of Fab' or SCA was added to sodium phosphate buffer (pH 7.2) to obtain a final concentration of 0.1 M and placed in a 12 × 75-mm glass tube coated with 20 µg of Iodo-Gen followed by 0.5 mCi of Na¹²⁵I or Na¹³¹I (DuPont-NEN, Boston, Mass). After a 2-minute incubation at room temperature, the protein was removed from the insoluble Iodo-Gen, and the unincorporated iodine was separated from the antibody by gel filtration through Sephadex G-25 (10-mL column). The labeled antibody in the void volume was pooled. The iodination protocol yielded labeled IgG and fragments with specific activities of approximately 3 µCi/µg with up to 60% of the input iodine bound to the protein.

SDS-PAGE

The MAB B6.2 Fab' and SCA preparations were analyzed for purity and integrity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The labeled antibodies were evaluated with and without reduction by 2-mercaptoethanol. Electrophoresis was performed according to the method of Laemmli (17) with a 15% polyacrylamide gel (16 × 14 cm) with a stacking gel of 3% acrylamide. Radiolabeled antibodies were detected by autoradiography with x-ray film (XAR film, Kodak, Rochester, NY) and intensifying screens (Lightning-Plus, DuPont, Wilmington, Del) at -70 °C.

Solid-phase Radioimmunoassays

The immunoreactivity of the MAB B6.2 IgG, Fab', and SCA was assessed in a competitive radioimmunoassay with tumor extracts. MAB samples were serially diluted in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and added to plates containing 2 µg/well of an extract of a human breast tumor metastasis with 100,000 cpm of ¹²⁵I-labeled B6.2 IgG or Fab'.

¹Johnson S et al.: manuscript in preparation.

²Pantoliano M et al.: manuscript in preparation.

Following an overnight incubation at 4 °C, the plates were washed and wells were counted. The percent inhibition was compared on a molar basis to that for purified B6.2 IgG, which served as a standard.

The immunoreactivity of the radiolabeled B6.2 preparations was assessed with a solid-phase radioimmunoassay with an extract of a human breast tumor metastasis as a positive control and an extract of normal human liver as a negative control. Twenty micrograms of each extract in 50 μ L of PBS was added to each well of 96-well polyvinyl microtiter plates and allowed to dry. The microtiter plates were treated with 100 μ L of 5% BSA for 1 hour at 37 °C to minimize nonspecific protein absorption. The BSA was removed, and varying amounts of radiolabeled antibody (in 50 μ L) were added. Following an overnight incubation at 4 °C, the unbound Ig was removed by washing the plates with 1% BSA in PBS. The bound radioactivity was detected by cutting the individual wells from the plate and measuring the radioactivity in a well-type NaI gamma counter (Pharmacia LKB).

Tumor Growth in Athymic Mice

Female athymic mice (nu/nu), obtained from the Charles River Laboratories (Wilmington, Mass) at 4–6 weeks of age, were injected subcutaneously on the back with 1×10^6 human colon carcinoma cells (LS-174T) (18) or human melanoma cells (A375) (19) (0.2 mL) under an approved protocol. Animals were used for biodistribution studies approximately 2 weeks postinoculation when they had tumors measuring 0.5–0.8 cm in maximal diameter.

Biodistribution Studies

Tumor-bearing mice were injected in the tail vein with approximately 0.5 μ Ci of 125 I-B6.2 SCA and 0.5 μ Ci of 131 I-B6.2 Fab'. Mice (three per data point) were killed by exsanguination. Blood, tumor, and all the major organs were collected and wet-weighted with an analytical balance, and radioactivity was measured in a γ -scintillation counter. The percentage of the injected dose per gram (%ID/g) for each organ was determined and tissue to blood ratios and radiolocalization indices (%ID/g of tumor divided by %ID/g of normal tissue) were calculated.

To obtain pharmacokinetic data, we obtained blood samples from the tail vein at various times after intravenous administration of both the 125 I-B6.2 SCA and the 131 I-B6.2 Fab'. Data on averages of three studies of three mice per study are presented.

Results

MAB B6.2 (11) and the LS-174T human colon carcinoma xenograft grown in athymic mice were used as the model system for the studies described here. MAB B6.2 recognizes a 90-kilodalton glycoprotein on the surface of human carcinomas as well as some normal tissues (12,13). While MAB B6.2 is not useful for anticancer applications in patients, it is one of the few MABs for which all three conventional forms (IgG, F(ab')₂, and Fab') have been well characterized (14,20) as to in vitro binding, in vivo tumor binding, and pharmacokinetics and whose DNA sequences of the V_H and V_L region are known. We describe here the first in vivo application of an SCA, its pharmacokinetic

properties, and the demonstration that it indeed is stable in vivo and has the ability to efficiently target a human tumor.

The B6.2 SCA used in these experiments is designated B6.2/212, where 212 refers to the novel specific peptide linker used. The B6.2/212 protein was produced in *E. coli* from a gene in which the sequence coding for the B6.2 V_L is connected to the B6.2 V_H coding sequence (16) via a DNA segment coding for the 212 linker (fig. 1). The 212 linker has the amino acid sequence GSTSGSGKSSEGKG and extends from L107 to H1 (Kabat numbering system). We found five errors in the published (16) V_H chain sequence and these were corrected in our construction. The cloning and sequencing of the correct B6.2 V_H and V_L regions will be reported elsewhere.¹ The properties of the 212 linker and the detailed methodology for the renaturation and purification of the B6.2/212 SCA by cation exchange high-pressure liquid chromatography will be reported elsewhere.²

As seen in figure 2, the SCA migrated as a homogeneous entity of 25 kilodaltons in SDS-polyacrylamide gels with or without reduction with 2-mercaptoethanol. A competitive radioimmunoassay using 125 I-labeled B6.2 IgG binding to a human breast tumor extract containing the B6.2 reactive antigen showed that the B6.2 Fab' and SCA competed similarly, both at approximately threefold the concentration of that of the intact IgG (fig. 3A). In a competitive radioimmunoassay using 125 I-labeled B6.2 Fab' binding to the tumor extract, Fab', IgG, and SCA forms of MAB B6.2 all competed similarly (fig. 3B), indicating equal in vitro antigen-binding potential of the Fab' fragments and SCA. Direct binding assays involving binding to a human breast tumor extract in solid-phase radioimmunoassays supported this point (data not shown). Affinity constant determinations (21) revealed a K_d of $4.3 \times 10^8 M^{-1}$ for the B6.2 IgG, $2.6 \times 10^8 M^{-1}$ for the Fab', and $3.2 \times 10^8 M^{-1}$ for the SCA (table 1).

To compare clearance rates and biodistribution, we administered preparations of 125 I-labeled SCA and 131 I-labeled Fab' forms of MAB B6.2 intravenously to mice. As seen in figure 4, a much more rapid blood clearance was observed for the SCA; 50% of the SCA was out of the blood pool by 3 minutes after administration, and 80% was removed by 15 minutes. The shape of the clearance curve for the SCA was determined to best fit that of a two-compartment clearance model (22), with an alpha phase $T_{1/2}$ (believed to be the clearance of molecules from the blood into the extravascular spaces) of 2.4 minutes and a beta phase $T_{1/2}$ (believed to be the clearance of molecules from the blood to nonextravascular spaces or out of the body) of 168 minutes (2.8 hr) (table 1). By contrast, the MAB B6.2 Fab' alpha phase $T_{1/2}$ is 14.8 minutes (table 1), and beta phase $T_{1/2}$ is 450 minutes (7.5 hr). Previous studies have shown the alpha phase $T_{1/2}$ values for MAB B6.2 IgG and F(ab')₂ to be 1.9 and 0.5 hours, respectively, and the beta phase $T_{1/2}$ values to be 41 and 14 hours, respectively (14,20). Whole-body clearance analyses (table 2) also demonstrated a very rapid SCA clearance, thus indicating that the SCA was not only leaving the blood pool but was not being retained in extravascular spaces and/or individual organs.

Experiments were then conducted to determine if the SCA construct retained its antigen-binding ability in vivo and had the ability to target human tumor xenografts. Comparisons of these properties for SCA versus Fab' were carried out by the co-administration of 125 I-labeled SCA and 131 I-labeled Fab' systemically in mice bearing the subcutaneous LS-174T human colon

GAATTCGGGGGATCTCTCACTACCAACAATGCCCCCTGCAAAAAATAAATTCATATAAAAAACATAC
 EcoRI
 O_L P_R SD (cro)
 AGATAACCATCTGCGGTGATAAATTATCTCTGCGGTGTTGACTATTTTACCTCTGGGGTGAZAAATGGTTGCATGTACTAAGGAGGTT
 HincII
 ompA signal -10 -1
 Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala Thr Val Ala Gln Ala
 GTATCGATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG GCC
 ClaI
 B6.2 V_L 5 10 15 20
 Asp Val Val Met Thr Gln Ser Gln Lys Phe Met Ser Thr Ser Val Gly Asp Arg Val Ser
 GAC GTC GTG ATG ACC CAG TCT CAA AAA TTC ATG TCC ACA TCA GTG GGA GAC AGG GTC AGC
 AarII
 25 30 35 40
 Val Thr Cys Lys Ala Ser Gln Asn Val Val Thr Asn Val Ala Trp Tyr Gln Gln Thr Pro
 GTC ACC TGC AAG GCC AGT CAG AAT GTG GTC ACT AAT GTA GCC TGG TAT CAA CAG ACA CCA
 45 50 55 60
 Gly Gln Ser Pro Lys Ala Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Ser Gly Val Pro Asp
 GGA CAA TCT CCT AAA GCA CTG ATT TAC TCG GCA TCC TAC CGG TAC AGT GGA GTC CCT GAT
 65 70 75 80
 Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Asn Val Gln Ser
 CGC TTC TCA GGC AGT GGA TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AAT GTG CAG TCT
 85- 90 95 100
 Gly Asp Leu Ala Glu Tyr Phe Cys Gln Gln Tyr Asn Ser Tyr Pro Leu Thr Phe Gly Ala
 GGA GAC TTG GCA GAG TAT TTC TGT CAG CAA TAT AAC AGC TAT CCT CTC ACG TTC GGT GCT
 105 212 Linker
 Gly Thr Lys Leu Glu Leu Lys Gly Ser Thr Ser Gly Ser Gly Lys Ser Ser Glu Gly Lys
 GGG ACC AAG CTT GAG CTG AAA GGT TCT ACC TCT GGT TCT GGT AAA TCT TCT GAA GGT AAA
 Hind III
 B6.2 V_H 5 10 15
 Gly Gln Val Thr Leu Lys Glu Ser Gly Pro Gly Ile Leu Lys Pro Ser Gln Thr Leu Ser
 GGT CAA GTT ACT CTT AAG GAG TCT GGC CCT GGG ATA TTG AAG CCC TCA CAG ACC CTC AGT
 AII
 20 25 30 35 35A 35B
 Leu Thr Cys Ser Phe Ser Gly Phe Ser Leu Ser Thr Ser Gly Met Gly Val Gly Trp Ile
 CTG ACT TGT TCT TTC TCT GGG TTT TCA CTG AGC ACT TCT GGT ATG GGT GTA GGC TGG ATT
 40 45 50 55
 Arg Gln Pro Ser Gly Lys Ser Leu Glu Trp Leu Ala His Ile Trp Trp Asn Asp Glu Arg
 CGT CAG CCT TCA GGA AAG AGT CTG GAG TGG CTG GCA CAC ATT TGG TGG AAT GAT GAG AGG
 60 65 70 75
 Tyr Tyr Asn Pro Ser Leu Lys Asn Gln Leu Thr Ile Ser Lys Asp Thr Ser Arg Asn Gln
 TAC TAT AAC CCA TCC CTG AAG AAC CAG CTC ACA ATC TCC AAG GAT ACC TCC AGA AAC CAG
 80 82A 82B 82C 85 90
 Val Phe Leu Lys Ile Thr Ser Val Asp Thr Ala Asp Thr Ala Thr Tyr Tyr Cys Ala Arg
 GTA TTC CTC AAG ATC ACC AGT GTG GAC ACT GCA GAT ACT GCC ACT TAC TAC TGT GCT CGA
 95 100J100K 105 110
 Ser Pro Arg Gly Tyr Phe Asp Tyr Trp Gly His Gly Thr Thr Leu Thr Val Ser * *
 TCC CCT AGG GGG TAC TTT GAC TAC TGG GGC CAC GGC ACC ACT CTC ACA GTC TCC TAATAAGGATTC
 BamHI

Figure 1. The B6.2/212 SCA gene fused to the ompA signal sequence under the control of the O_L/P_R promoter. The gene contains a synthetic V_L (16) and a cDNA copy of the V_H region, joined by a sequence designated linker 212.¹ Restriction sites used to join the various genetic elements are indicated. The ompA signal sequence provides an effective translation start and is then efficiently removed. The translated variable-region sequences are numbered using the Kabat system.

carcinoma xenografts. At various time points after injection, blood, tumor, and normal organs were analyzed to determine the amount of each radionuclide per gram of tissue. As seen in table 3, similar values for percent injected dose per gram (%ID/g) were obtained in the tumor for SCA and Fab' at one-half hour, 4 hours, and 6 hours; at 24 hours, slightly higher values were obtained with the Fab'. Differences observed for kidney uptake will be discussed. When one examines the %ID/g values for blood and well-vascularized organs such as liver and spleen, one observes that the values for the SCA are much lower than those for the Fab'. Thus, the radiolocalization index (RI) values (%ID/g tumor divided by %ID/g of normal organ) demonstrate that tumor to normal tissue ratios for the SCA are similar to, or for some organs, better than those for Fab' at all time points, with extremely good values at 6 hours and 24 hours after SCA or Fab'

administration (table 4). As an additional control, ¹²⁵I-labeled SCA was administered to athymic mice bearing a human melanoma (A375) xenograft, which lacks the B6.2 reactive antigen. RI values were all below 1.5, demonstrating the absence of nonspecific uptake by a tumor xenograft that does not contain the B6.2 SCA reactive antigen (table 5).

Particular attention should be given to the comparative kidney values for the SCA and the Fab' in the colon carcinoma-bearing animals. Previous studies using Fab' or F(ab')₂ fragments of several MAbs in mice and humans have shown that these antibody forms are accumulated in the kidney at a rate greater than would be predicted by simple elimination from the body (14,23). This finding was also observed in the current study for the MAb B6.2 Fab' in which there was 132% ID/g in the kidneys one-half hour following Fab' administration, and percentages

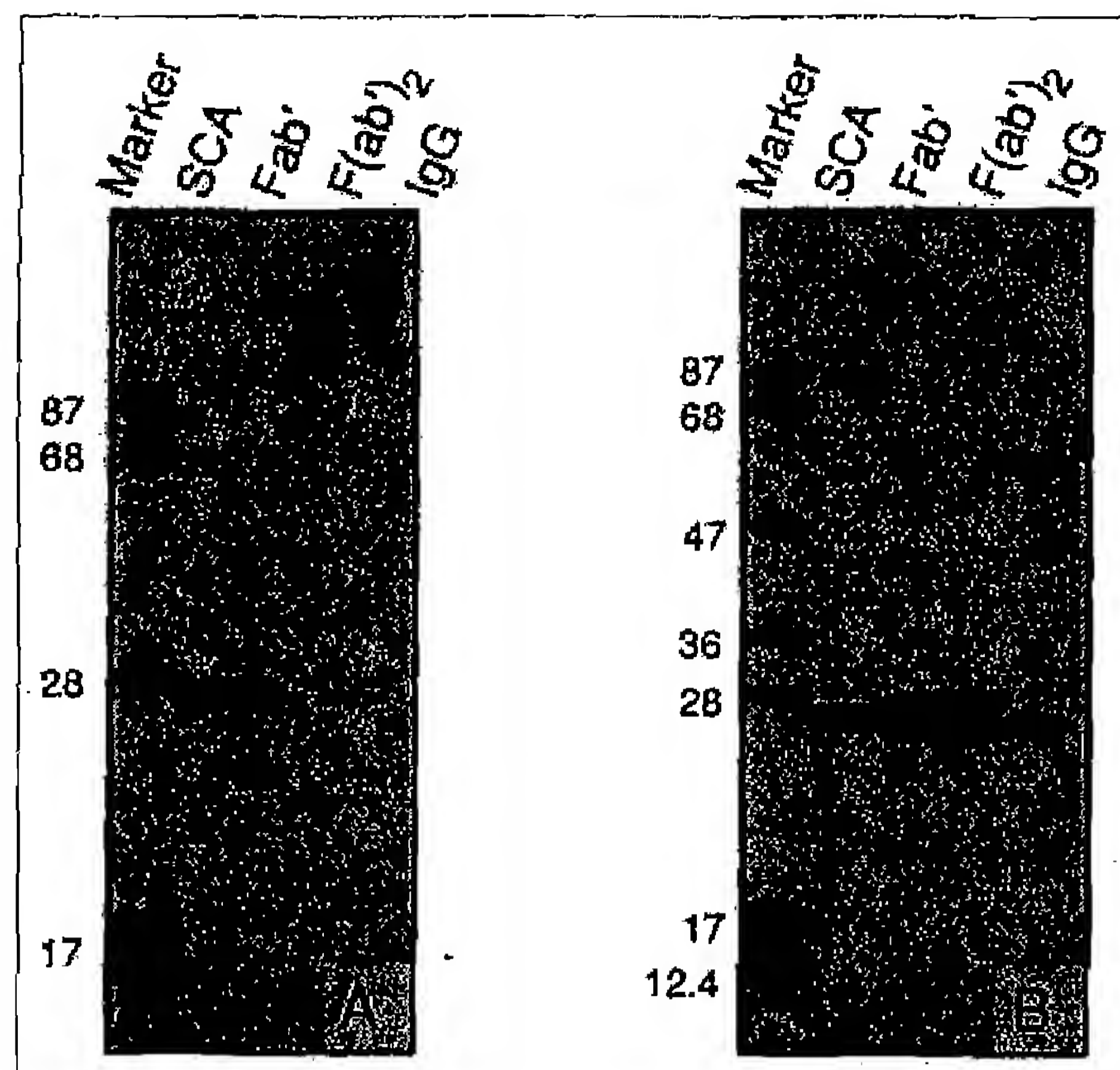


Figure 2. Purified MAb B6.2 IgG, its fragments, and the B6.2 SCA were analyzed by SDS-PAGE on a 15% polyacrylamide gel with (B) and without (A) reduction with 2-mercaptoethanol. The SCA migration in the gels was consistent with a molecular mass of ≈ 25 kilodaltons. The Fab' fragment separated into its two component chains of ≈ 25 kilodaltons, because of its previous reduction and alkylation. The MAb B6.2 IgG and F(ab')₂ fragment ran as ≈ 150 and 100 kilodaltons, respectively, without reduction with 2-mercaptoethanol. After reduction, the IgG separated into its heavy and light chains and the F(ab')₂ molecule separated into its Fd and light chains.

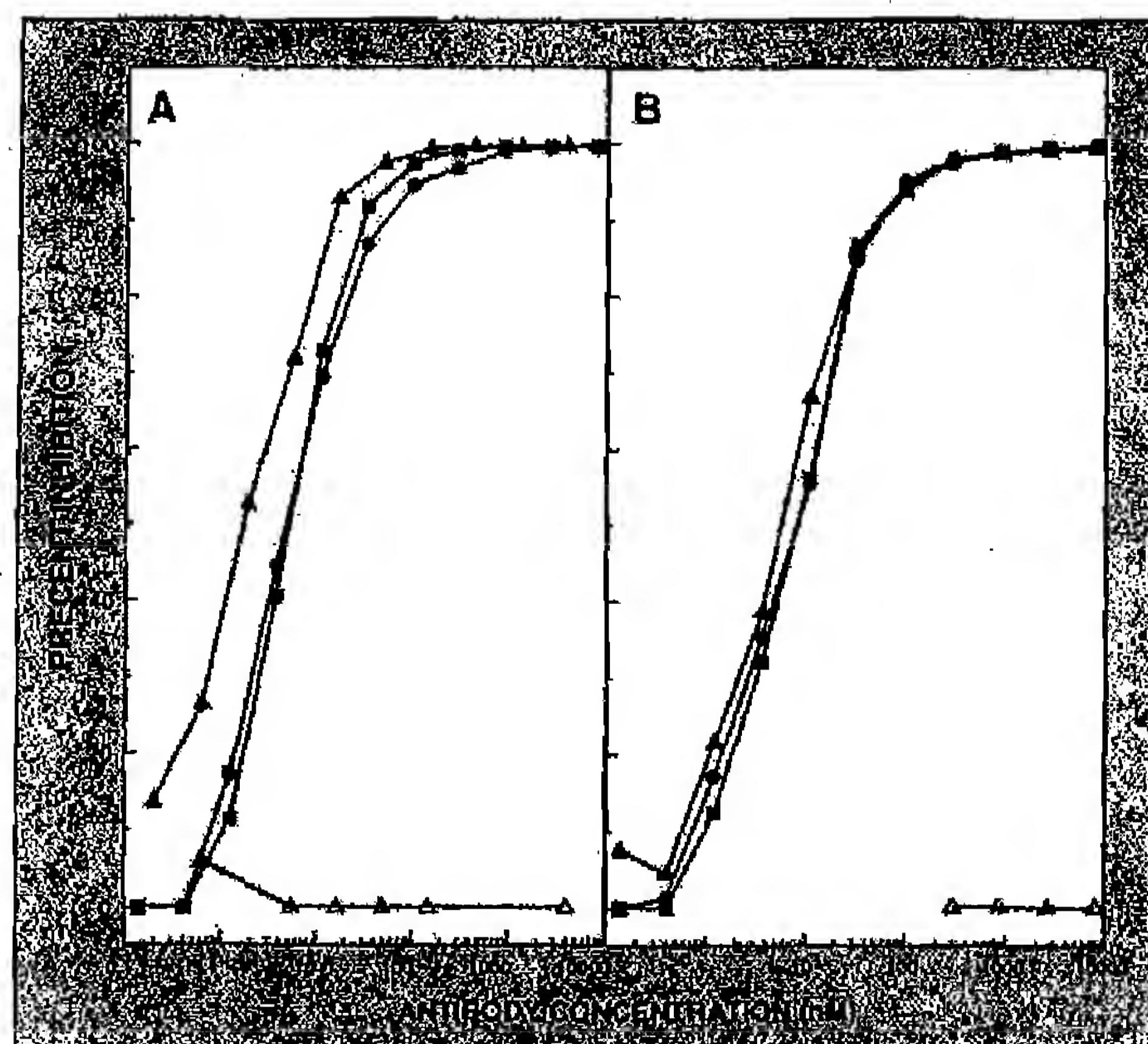


Figure 3. Analysis of MAb B6.2 IgG, Fab', and SCA in competitive radioimmunoassay. ¹²⁵I-B6.2 IgG was added to wells containing 2 μ g of a human breast tumor extract with different nanomolar amounts used as competitors, as described in the Materials and Methods section. (A) B6.2 IgG (solid triangles); B6.2 Fab' (solid circles); B6.2 SCA (solid squares), and MAb BL-3 control (lymphoma anti-idiotypic) MAb (open triangles). (B) Data from similar competitive radioimmunoassay using ¹²⁵I-B6.2 Fab', binding to breast tumor extract.

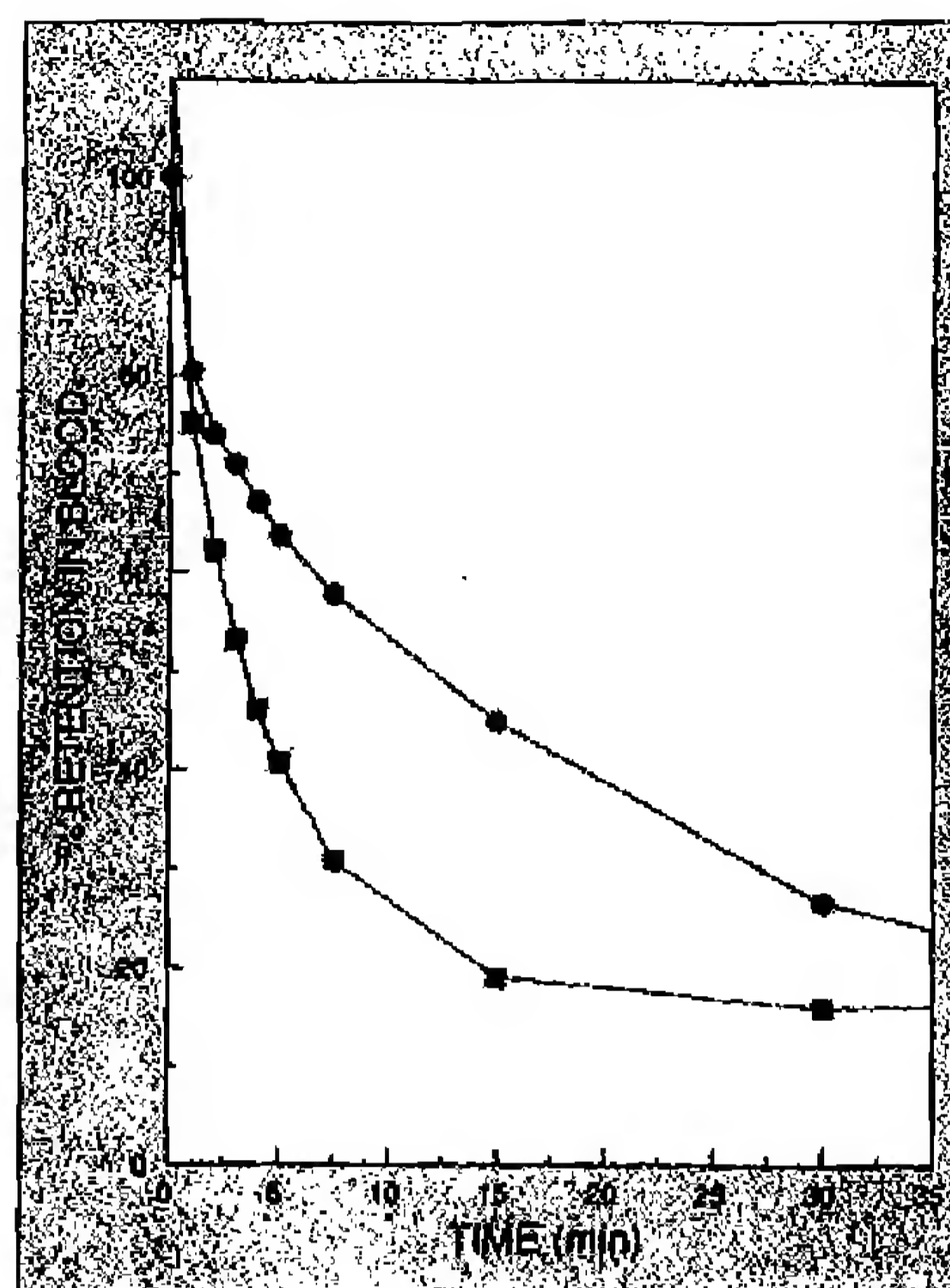


Figure 4. Pharmacokinetics of blood pool clearance of MAb B6.2 Fab' fragment (solid circles) and SCA (solid squares) in athymic mice bearing the LS-174T colon carcinoma xenograft. ¹²⁵I-labeled SCA and ¹³¹I-labeled Fab' were co-injected intravenously in mice bearing subcutaneous tumor; blood samples were obtained at the times indicated. Each data point represents three separate experiments.

10-fold greater in the kidneys than in the tumor 6 hours after Fab' administration (table 3). This observation contrasts with the apparent rapid elimination of the SCA through the kidneys and the high tumor to kidney ratios at all time points after one-half hour following SCA administration.

Discussion

There are numerous reasons why SCAs may prove potentially useful for clinical applications in the management of cancer and other diseases in which conventional MAb or hyperimmune sera are now being used or are being considered for use. These reasons include:

Table 1. Affinity constants and blood clearance of MAb B6.2 IgG and fragments

Ig form	$K_D (M^{-1})$	$T_{1/2\alpha} (min)$	$T_{1/2\beta} (min)$
IgG*	4.3×10^8	114.0	2,460
Fab'	2.6×10^8	14.8	450
SCA	3.2×10^8	2.4	168

*The $T_{1/2}$ of the IgG is taken from reference (20).

Table 2. Comparison of whole-body clearance of SCA and Fab'

Time (hr)	%ID	
	SCA	Fab'
0.5	58.6*	100.0
1	43.9	87.5
4	25.4	64.7
6	17.3	43.3

*%ID in whole body at the indicated times following intravenous administration of ¹²⁵I-labeled SCA and ¹³¹I-labeled Fab' to athymic mice.

Table 3. %ID/g of MA b B6.2 SCA and Fab' in athymic mice bearing human colon carcinoma xenografts*

Specimen	SCA				Fab'			
	0.5 hr	4 hr	6 hr	24 hr	0.5 hr	4 hr	6 hr	24 hr
Tumor	6.89	5.18	3.05	0.91	4.61	5.00	3.35	1.60
Blood	6.65	2.72	0.49	0.13	6.40	3.01	0.99	0.30
Liver	3.39	1.14	0.24	0.08	2.44	1.42	0.61	0.20
Spleen	4.70	1.39	0.35	0.12	3.28	1.69	0.78	0.26
Kidneys	11.65	1.86	0.65	0.17	132.08	41.45	33.25	2.88
Lungs	7.82	2.70	0.54	0.37	5.55	2.56	0.86	0.41

*Values = %ID/g for average of three mice at time after SCA or Fab' administration.

Table 4. Radiolocalization index of MA b B6.2 SCA and Fab' in athymic mice bearing human colon carcinoma xenografts*

Specimen	SCA				Fab'			
	0.5 hr	4 hr	6 hr	24 hr	0.5 hr	4 hr	6 hr	24 hr
Blood	1.04	1.91	6.24	7.26	0.72	1.66	3.37	5.26
Liver	2.03	4.55	12.49	11.81	1.89	3.53	5.52	7.95
Spleen	1.46	3.72	8.74	7.70	1.40	2.95	4.28	6.16
Kidneys	0.59	2.79	4.71	5.38	0.03	0.12	0.10	0.55
Lungs	0.88	1.92	5.63	2.45	0.83	1.95	3.90	3.89

*Values = radiolocalization index (%ID/g of tumor divided by %ID/g of normal tissue) for average of three mice at time after SCA and Fab' administration.

Table 5. Specificity of in vivo tumor localization of a single-chain antigen-binding protein*

Specimen	LS-174T colon carcinoma		A375 melanoma	
	4 hr	17 hr	4 hr	17 hr
Blood	1.91	7.41	0.87	1.10
Liver	4.55	9.27	1.82	1.47
Spleen	3.72	8.81	1.07	1.50
Kidneys	2.79	4.36	0.20	0.56
Lungs	1.92	1.85	0.76	0.66

*Values = radiolocalization index (%ID/g of tumor divided by %ID/g of normal tissue) for average of three mice with LS-174T colon carcinoma xenograft or A375 melanoma xenograft at time after SCA administration.

- The very rapid clearance from the blood pool and whole body demonstrated here makes SCA molecules particularly attractive for coupling to radionuclides and some drugs in an attempt to reduce toxic effects in normal tissues.
- Our current data demonstrate that the unwanted accumulation of Fab' and F(ab')₂ in the kidneys is not seen with the SCA. This is potentially important for detection of tumors in the peritoneal cavity and for reduction of potential renal toxic effects associated with the use of drug- or radionuclide-conjugated Ig fragments.
- The small size of SCAs should improve their capacity for rapid and evenly distributed penetration through tumors and other target tissues.

- SCAs should have reduced immunogenicity because they do not contain C_H2 and C_H3 domains of intact Igs, nor the C_H1 or C_L domains found in Fab' or F(ab')₂ fragments. The absence of a C_H1 and C_L domain may also reduce the anti-allotype responses.
- Because Fab' or F(ab')₂ fragments are generated through proteolysis, they are often difficult to produce in immunoreactive form for in vivo use. In contrast, SCAs, which are synthesized from recombinant genes in *E. coli*, should be easier to make reproducibly in active form.
- Costs associated with the production, purification, and quality control of clinical grade SCAs should be greatly reduced, compared with those of conventional MAbs or MA b fragments. The SCA used in this study was produced from *E. coli* at a level of 6 mg of SCA per liter of broth, resulting in recovery of approximately 3 mg of homogeneously pure SCA per liter. Moreover, contamination with mammalian DNA (i.e., oncogenes), mammalian retroviruses, or murine adventitious viruses such as hepatitis is not a problem as it is with conventional MAbs produced in mammalian cells. In addition, there is no need to use the costly procedure of generating a proteolytic fragment from an intact IgG under the strict guidelines required for use as a clinical reagent.
- Because SCA molecules are so small, it may be possible, for more efficient therapeutic and/or diagnostic applications, to add regions of the Ig molecule responsible for effector- or complement-mediated functions or to add drugs (10) or specific combining sites for drugs and radionuclides (i.e., bifunctional chelates).

Conclusions

Studies reported here thus demonstrate that SCA molecules can now be seriously considered for clinical applications.

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